Interpretation of Laboratory Tests for Diagnosis of Sickle Cell Anemia Clinician's Perspective

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Abstract

Sickle cell disorder is a hereditary hemolytic anemia. Considering India' population size more than 50% of world' sickle cell anemia cases resides in India . Vidarbha region is considered as high risk zone for sickle cell disease Since this disorder has created social impact it is mandatory for clinicians to provide the correct diagnosis With the help of High Performance Liquid Chromatography (HPLC) prognostic outcome can be predicted. This article highlights the laboratory diagnosis of sickle cell anemia with reference to interpretation of these tests from clinician's perspective

Key Words : laboratory tests , sickle cell disease,

Sickle cell disorder is a hereditary hemolytic anemia. This disorder results from genetic mutation substituting thymine for adenine , which results in replacement of glutamic acid by valine in 6th position from N terminal on β chain. As a result of this abnormal hemoglobin is produced –Hb S. This abnormal hemoglobin undergoes polymerization in hypoxic state and gives peculiar sickle shape to the red blood cells. Sickle cell disorder is observed as heterozygous state / carrier(Hb AS), homozygous state / sickle cell anemia (Hb SS) and as double heterozygous states like Sickle - β Thalassaemia,or Sickle –Hb D Punjab etc.

More than 50% of world's sickle cell anemia cases resides in India .The incidence of sickle cell anemia in mahar ,kunbi and teli community as reported by Shukla and Solanky is 22.2 %,9.4 % and 11.3 % respectively[1]. Since Vidarbha has higher percentage of these communities as compared to rest of Maharashtra, Vidarbha region is considered as high risk zone. Government of Maharashtra initiated sickle cell moment and decided to carry out sickle cell screening programme at remote places. Responsibility of mass screening is handed over to public health department whereas management of serious cases, critical care, prenatal diagnosis was assigned to tertiary care centers like government medical colleges. Health awareness programmes are being conducted by Non Government Organizations utilizing multimedia for mass communication.. Sickle cell anemia is remarkably variable in its clinical expression even among affected members of the same family. Whereas many individuals have recurrent ,severe complications others enjoy a relatively benign virtually symptom free course. Since this disorder has created social impact it is mandatory for clinicians to provide the correct diagnosis With the help of High Performance Liquid Chromatography (HPLC) prognostic outcome can be predicted. This article highlights the laboratory diagnosis of sickle cell anemia with reference to interpretation of these tests from clinician' perspective..

Laboratory investigations for the diagnosis of sickle cell disorders are as follows

1. Sickling test
2. Solubility test
3. Hemoglobin electrophoresis
4. High Performance Liquid Chromatography (HPLC).
5. Immunofixation
6. Isoelectric focusing
7. Globin chain chain electrophoresis.
8. PCR /DNA analysis /gene mapping

Sickling test, Solubility test and Hemoglobin electrophoresis are commonly used. High Performance Liquid Chromatography (HPLC) is available in some

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of the medical colleges.

Immunofixation,Isoelectric focusing,,Globin chain chain electrophoresis, and PCR /DNA analysis /gene mapping are available at research institutes.

Sickling Test

Simple test easy to perform and highly sensitive if interpreted against the proper controls.It is based on inducing sickling in vitro by removing oxygen from red blood cells..At low oxygen Hemoglobin S is less soluble and induces sickling. .Reducing agent is added to remove oxygen from blood. In this test 2% freshly prepared sodium metabisulphite is mixed with drop of blood .This drop is covered with cover slip and sealed with petroleum jelly or nail polish.The glass slide is observed under microscope after one hour for sickled red cells[2] **(Fig I).**

Advantages

- The test is simple and cheaper as only one chemical is required.
- This test is useful in patients with severe anemia.

Limitations

- This test can not differentiate between heterozygous (AS) and homozygous (SS) state
- Even though sickling takes place as early as ½ hr, the heterozygote may take 12 to 24 hrs for sickling to occur.
- This test requires microscope and expert interpretation especially in the presence of anisopoikilocytosis.
- This test is not useful for cord blood screening..
- Hemolyse samples or improper sealing of glass slide may lead to erroneous results.

Solubility test

Solubility test is a rapid method for screening of sickle cell anemia. This is a test of choice for mass screening.

Sickle hemoglobin (Hb S) (reduced state) in high molarity phosphate buffer solution creates turbidity as it is insoluble. Adult hemoglobin in contrast is soluble in this buffer . Insoluble hemoglobin S creates turbidity and hence lines on the card can not be seen **(Fig II)** through the test tube. This is a naked eye test and results are available in 10 minutes. It is very essential to run positive and negative control with every batch of samples to be tested[2]

Advantages

- This test is rapid (10 min) and does not require microscope.
- This test is suitable in poulation screening camps wherein the high risk population is separated into positive and negative groups . the positive cases can further be subjected for further analysis like electrophoresis and HPLC techniques.

Limitations

- This test can not differentiate between heterozygous (AS) and homozygous (SS) state
- False negative results can be obtained in presence of severe anemia ,and on cord blood samples. (cord blood contains high percentage of Hb F)
- False positive results can be obtained in severe leucocytosis as in CML or leukemoid reactions, in multiple myeloma and in presence of other sickling hemoglobins like Hb C – Harlem or Hb S –Travis.

Hemoglobin Electrophoresis (Hb Ep)

Hemoglobin electrophoresis is a technique used to separate different hemoglobins from blood so that patient can be categorized into heterozygous or homozygous state..Hemoglobins separate out as they carry different electrical charges and migrate at various positions from anode to cathode. Routinely hemoglobin electrophoresis is carried out at alkaline pH on cellulose acetate[2]

Interpretation of Hb Electrophoresis (Fig III)

- Normal healthy adult has predominant hemoglobin HbA followed by very small fraction of Hb A2 and Hb F **This is reported as AA pattern on electrophoresis**.
- At birth there is predominant F hemoglobin with variable quantity of Hb A. **On Ep it is reported as Hb FA pattern**
- Sickle cell carrier / heterozygous state – Two hemoglobins are seen Hb A and Hb S **On Ep it is reported as Hb AS pattern**
- Sickle cell anemia / homozygous state –Two hemoglobins are seen Hb S and Hb F **On Ep it is reported as Hb SS/SF pattern**

DD of AS pattern on Ep

1) Sickle cell trait
2) Hb SS with recent blood transfusion
3) $S\beta^+$ thalassemia
4) Hb D Punjab trait
5) Hb Lepore trait
6) Hb D Iran trait

Sickling test will be positive in conditions like sickle cell anemia (Hb SS) and sickle cell carrier and double heterozygous with Hb S like HbS -E , HbS-D etc.

DD of SS pattern on Ep

1) Sickle cell anemia (homozygous)
2) Double heterozygous state HbS –HbD Punjab In such cases Hb Ep has to be repeated at acidic pH / by HPLC analysis
3) Homozygous Hb D Punjab
4) Double heterozygous state HbS–β Thalassaemia. In such cases In such cases one of the parents will be β Thalassaemia trait and other will be Sickle cell trait.
5) Double heterozygous state HbS –HPFH (Hereditary Persistence of Fetal Hemoglobin)

Advantages of Hb Ep

Hb Electrophoresis can easily differentiate between carrier (AS) and homozygous (SS) state.

Limitations

- HbS and HbD Punjab cannot be separated on electrophoresis. HbD Punjab cases are observed in Maharashtra especially in sindhi and Punjabi communities. Hb D homozygous have mild hemolytic anemia as compared to HbSS. Hence it is very necessary to diagnose these cases. If Hb Electrophoresis and Sickling test results are correlated together HbD can be diagnosed as sickling test will be negative in Hb D Punjab.
- Similarly Hb S, D Iran ,Q India ,Lepore and G resolves at the same position cannot be separated on electrophoresis hence it is necessary to use other methods like HPLC.
- Hb A_2 and Hb E can not be differentiated on electrophoresis.
- Ep pattern of sickle cell anemia (HbSS) will change to HbAS on blood transfusion. This change will remain for about 2-3 months.

Automated High Performance Liquid Chromatography HPLC

This is an ion exchange chromatography technique where abnormal hemoglobins can be identified and quantitated at the same time. Fully automated machine by Bio rad company is in use with some of the medical colleges in Maharashtra. When hemolysate (solution of hemoglobin) containing abnormal hemoglobin is adsorbed onto the resin column ,the rate of elution of different hemoglobins is determined by the pH and ionic strength of buffer. The different hemoglobins pass through the light detector and are recorded on an integrating computer system. It is displayed in the form of chromatogram of absorbance verses time (Fig III). To aid in the interpretation windows have been established based on the characteristic retention time .retention time is the time from injection of sample to the apex of a hemoglobin peak. each hemoglobin has a characteristic retention time[2].

Interpretation[2]

It is important to recollect following facts Normal healthy adults show three hemoglobins Hb A , Hb A_2 and HbF.

Normal Healthy adult (Fig IV) Hb A > Hb A_2 >HbF

- Hb A 96 -98 %
- Hb A_2 <3.5 %
- Hb F <1 %

Interpretation of common chromatograms on HPLC (See Photgraphs)

Sickle cell trait (Hb AS) (FigV)

Hb A 62-65 %

Hb S 35- 38%

Hb A_2 < 3.5%

Hb F <1 %

Ø In Sickle cell trait Hb A > Hb S. Post transfusion SS can sometimes be confused with AS but here Hb S > Hb A.

Ø Normal person (Hb AA) if receives blood from a sickle cell carrier will show a small 6 to 10% of Hb S for few days and this should not be considered as sickle trait.

Ø From percentage of Hb S association of α

thalassemia can be diagnosed as Sickle cell trait.in association with α thalassemia show decreased percentage of Hb S. Sickle cell trait with α+ thalassemia will have Hb S percentage 28- 35 while Sickle cell trait with α^0 thalassemia will have Hb S percentage 20- 30. Sickle cell trait.in association with α thalassemia have less severely impaired urinary –concentration ability hence better prognosis as compared AS without α thalassemia[3,4]

Ø **Sickle cell anemia (Hb SS) (Fig VI)**

Hb S 88 -93 %

Hb A 0

Hb A_2 < 3.5%

Hb F 5 -10%

Ø Hb S is the predominant hemoglobin in these cases followed by Hb F.

Ø In Indian sickle cell cases Hb F % varies 15 to 25 %

Ø If Hb F % is more ie 20-30 % association of HPFH can be considered. Family study will confirm the final diagnosis. In such cases one of the parents will be HPFH trait and other will be Sickle cell trait.

Ø Sickle cell anemia produces symptoms by the age of 2 years. The patient presents with a variety of symptoms like vasoocclusive crisis ,hand foot syndrome, painful crisis, acute chest syndrome CNS manifestations etc Remarkable variability in clinical expression of sickle cell anemia is because of some modulators like HbF levels , the presence of α thalassemia, the β gobin cluster haplotype and gender. In eastern province of Saudi Arabia and in Kuwait ,Iran , India and the West Indies mild disease is associated with Hb F levels of 15 to 30 %[5]

Double heterozygous states

Ø **Sickle cell – β^0 Thalassemia (Hb S - β^0)**

Hb S 88 -93 %

Hb A 0

Hb A_2 > 3.5%

Hb F 5 -10%

§ Sβ^0thalassemia resemble Hb SS clinically and on electrophoresis . It is higher percentage of **Hb A_2 (4-6 %)** which determines the associated thalassemia.

§ Sβ^0thalassemia cases have a slightly higher hemoglobin level and a smaller MCV (65 to 75 fl) as compared to SS cases.

§ Family screening is indicated in such cases.

Ø **Sickle cell – β^+ Thalassemia (Hb Sβ^+)**

Hb S 50 -93 %

Hb A 3-30 %

Hb A_2 > 3.5%

Hb F 1-10%

§ **Sβ^+** thalassemia can be misinterpreted as sickle cell trait on electrophoresis β^+ will produce some Hb A 3-30 %.

§ **Post transfusion SS** will also show similar findings but here **Hb A_2** will be normal as against ↑ **Hb A_2 in** Sβ^+ thalassemia.

Ø **Sickle cell- Hb E (Hb SE)**

Hb S > 30%

Hb A 0

Hb A_2 > 30%

Hb F ↑

§ **Hb E co elutes with Hb A_2 Hb E can be identified by the amount of Hb A_2 (15-40%).**

§ **Patients with Hb SE** may have mild anemia and microcytosis **.**

Ø **Sickle cell - Hb D (Hb SD)**

Hb S > 30%

Hb D > 30%.

Hb A_2 ↓

Hb F ↑

§ HPLC easily separate out HbS and D in contrast to Electrophoresis.

§ With HbD Punjab A_2 is decreased.

§ Hb SD Punjab patients may have mild hemolytic anemia and symptoms that mimic sickle cell anemia[5].

Ø **Sickle cell –HPFH (Hereditary Persistence of Fetal Hemoglobin) Hb S HPFH**

Hb S 65 --80%

Hb A 0

Hb A_2 <3.5 %

Hb F 20 -35%%

§ **Hb S HPFH** results in a heterogeneous disorder that is extremely mild and associated with pancellular distribution of Hb F. It is important to diagnose this condition as prognosis is extremely excellent[5]

Neonatal screening

Healthy New born baby[6] Hb F >Hb A > Hb A_2

- Hb F 80 %
- Hb A 20 %
- Hb A_2 0.5 %

New born screening is carried out to detect minor Hb components in the presence of large amounts of Hb F.HPLC can pick up the smallest quantity of Hb S in the cord blood or heel prick samples. Primary goal of newborn screening is reduce morbidity and mortality by identifying these infants at birth and proving ongoing care to them. Cord blood screening is advised when parents are sickle cell carriers. After provisional diagnosis of as having SS / AS retesting is done after 6-8 weeks of birth.

Conclusions:-

Correct diagnosis off sickle cell disease being a genetic disorder is very critical as this disorder is associated with social stigma. Combination of test is always advisable like sickling with electrophoresis or sickling with HPLC.Solubility test is usually used in population screening camps.

Electrophoresis is commonly used for conformation and typing of the disease as it is cheaper as compared to HPLC. However electrophoresis has its limitations.

Ideally HPLC should be done in all cases of hemoglobinopathy. Co- existence of disorders like βThalasamia, α thalassemia ,HPFH, Hb E and Hb D can be picked up by HPLC only . It is important to diagnose these conditions because of prognostic implications. HPLC is also used to moniter Hb F level when hydroxyurea therapy is implemented or when exchange transfusion is carried out. HPLC has very high precision and results are available in few minutes .When one patient is identified all the family members needs to be screened.

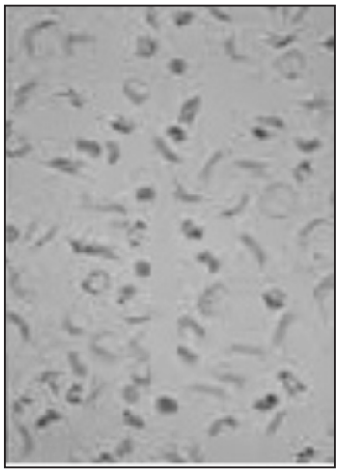


Fig I showing sickled red cells

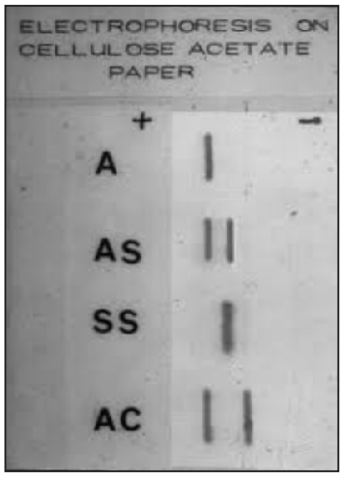
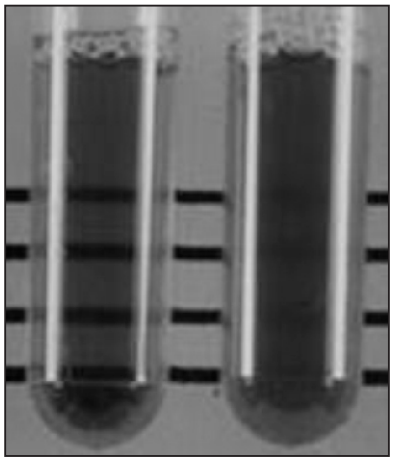
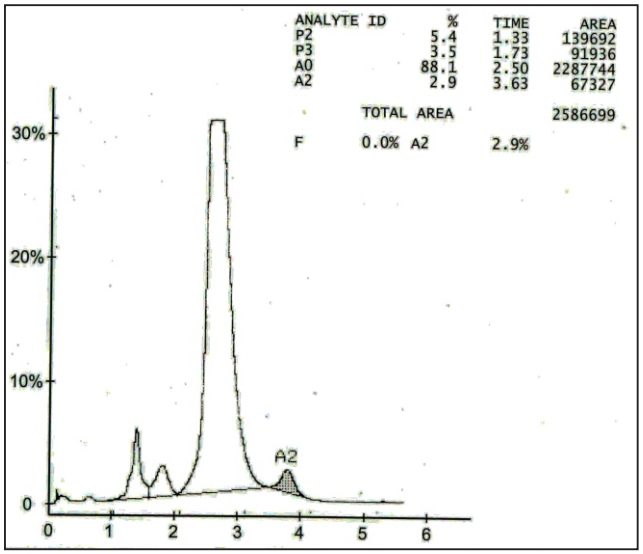


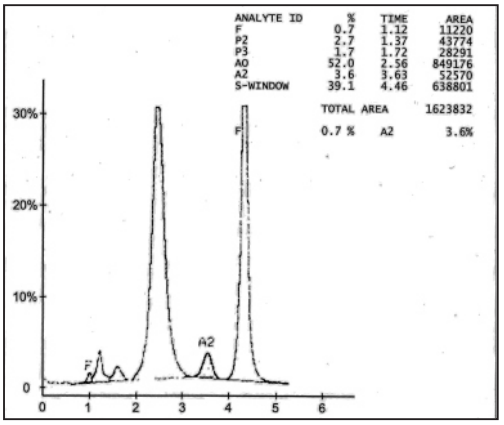
Fig II Showing solubility test.
Test tubes are held against lined card

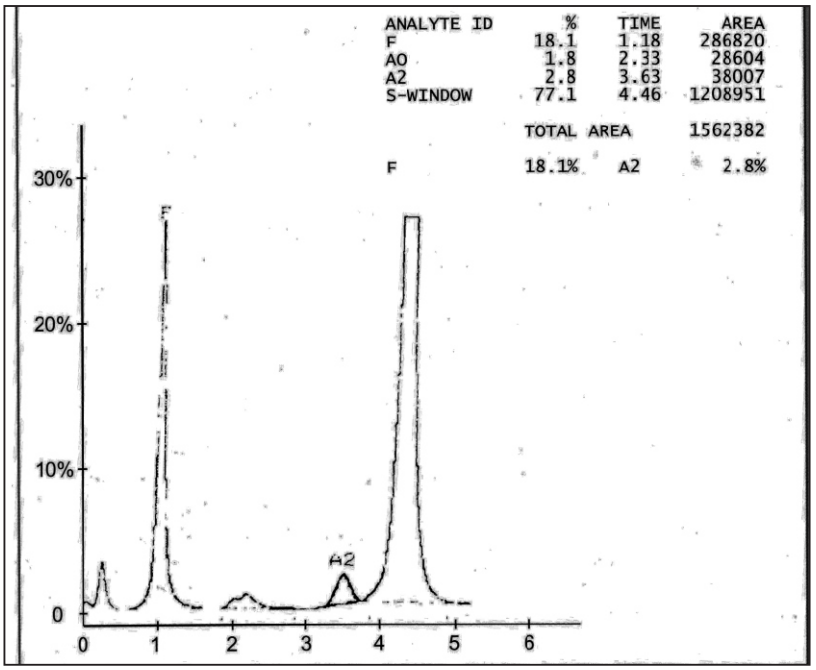


Negative Positive

Fig III showing hemoglobin electrophoresis at alkaline pH







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